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Estimation of carbon biomass and community structure of planktonic bacteria in Lake Biwa using respiratory quinone analysis

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Running title: Carbon biomass and community structure of bacteria in Lake Biwa

Abstract

The relationship between bacterial respiratory quinone (RQ) concentration and biomass was assessed for Lake Biwa bacterial assemblages to evaluate the utility of bacterial RQ concentration as an indicator of bacterial carbon. Biomass estimated from RQ concentration correlated well with that from cell volume, indicating that RQ concentration is an appropriate indicator of bacterial biomass. The estimated carbon content per RQ content (carbon conversion factor) of bacteria was 0.67 mg C nmol RQ^{-1}. Bacterial carbon biomass, which was estimated from RQ concentration using the conversion factor, ranged between 0.008 and 0.054 mg C L^{-1} (average: 0.025 mg C L^{-1}) at 5 m depth and between 0.010 and 0.024 mg C L^{-1} (average: 0.015 mg C L^{-1}) at 70 m depth. Ubiquinone-8-containing bacteria dominated the epilimnion and hypolimnion. Compared to the conventional image analysis, the bacterial RQ analysis is a less laborious way for simultaneous determination of bacterial biomass and community.

Introduction

Bacteria are numerically important components in the water columns of freshwater and marine systems. Many studies have demonstrated that the efficiency of recycling of matter depends on the bacterial metabolic activity, growth rate, and biomass (reviewed by Ducklow 2000; Azam and Malfatti 2007). As a result, bacteria are thought to be the major players in mineralization and biogeochemical organic matter transformations. Previous studies have reported that natural bacteria differ in terms of size distributions (Nagata 1986), metabolic state (Gasol et al. 1999), dissolved organic matter (DOM) utilization (Kirchman et al. 2004; Yokokawa and Nagata 2010), and growth rate (Yokokawa et al. 2004).

Since natural bacterial assemblages consist of various subgroups in terms of ecological and biogeochemical features, the phylogenetic and functional diversity of bacteria has been investigated in marine and freshwater environments. A majority of previous studies have discriminated bacterial communities on the basis of the 16S rRNA gene and with dramatic development of molecular biological techniques, the phylogenetic and functional diversity of bacteria has been revealed (Glöckner et al. 1999, 2000; Kirchman et al. 2004; Yokokawa et al. 2005). However, phylogenetically distinct bacterial groups do not always correspond to functional groups in matter cycling (Langenheder et al. 2005, 2006). The information about physiological discrimination of bacterial groups such as cellular components and nucleic acid content is quite limited, and the utility of physiological discrimination in bacterial community in the carbon cycling has not yet been fully understood.

Bacterial biomass is the fundamental parameter that directly links estimations of production, growth efficiency, and bioenergetics of the bacteriovores. Image analysis of bacterial cell volume is the most common tool used to estimate bacterial biomass, and this method has improved over several decades (Nagata and Watanabe 1990; Blackburn et al.

1998; Posch et al. 2009). However, image analysis is time-consuming, laborious, and involves many technical difficulties such as staining with fluorescent dyes (Posch et al. 2001; Straza et al. 2009) and artificial errors in cell volume measurement (Nagata and Watanabe 1990). A less time-consuming, less laborious, and more accurate method for bacterial carbon biomass measurement is needed to evaluate bacterial contributions to matter cycling in aquatic systems.

Respiratory quinone (RQ), including ubiquinone (UQ) and menaquinone (MK), are components of the electron transport chain located in the bacterial plasma membranes. Bacteria have diverse respiratory systems, and they can use more than 20 inorganic or organic redox pairs for energy production (Li 2010). Individual RQs differ in their preference for the electron acceptor for energy metabolism. In general, UQs are mainly produced for the oxygen and nitrate respiratory types because of the large midpoint potential between UQs and UQH_2 (+0.122 V), whereas MKs (-0.074 V) are mainly produced for the respiratory types with low-potential electron acceptors. As individual bacteria has only 1 dominant RQ type, RQs are potentially useful as specific biomarkers for discriminating between the biomass of bacterial subgroups with different types of energy metabolism (Collins and Jones 1981; Hedrick and White 1986; Villanueva et al. 2007). RQs have been shown to be appropriate biomarkers for tracing the bacterial biomass in various environments (Hedrick and White. 1986; Hiraishi 1999), as they are membrane lipids and not storage lipids, and undergo rapid degradation within hours or days after cell death (Hiraishi and Kato 1999). As these biomarkers directly indicate the bacterial biomass, improvement in the chemical determinations of bacterial biomass may provide a less time-consuming and more accurate method for estimating bacterial biomass in aquatic systems.

RQ typing, which discriminates bacterial subgroups based on differences in energy metabolism, may be appropriate for identifying useful functional units in ecological

matter-cycling studies. However, there is not much clarity on whether bacterial RQ concentration can be used as an indicator of bacterial biomass in water columns, although bacterial quinone concentrations in soil environments have displayed linear relationships with bacterial carbon concentration (Saitou et al. 1999) and cell abundance (Hiraishi et al. 2003). Thus, our major goal was to examine the utility of planktonic bacterial RQ analysis as a reliable method for carbon biomass estimation in Lake Biwa. Moreover, we also evaluated the reliability of RQ-based bacterial discrimination as a tool for planktonic bacterial community analysis.

Materials and Methods

Study site and sampling

Lake Biwa is a large (surface area, 674 km^2), deep (maximum depth, 104 m), monomictic, and mesotrophic lake located in the central part of Honshu Island, Japan. The sampling station for the present study was a pelagic station (35º 12′58″ N, 135º 59′55″ E; maximum depth 73 m), in the north basin of the lake. Samples were collected every month from June 2010 to March 2011. Depth profiles of water temperature were determined using a CTD probe (SBE 911 plus; Sea Bird Electronics). Samples were collected from 2 distinct layers at depths of 5 m (the epilimnion) and 70 m (the hypolimnion) with a 10-L acrylic water sampler and poured into 5- or 10-L polyethylene bags for RQ analysis. The samples were also collected with 5-L Niskin X bottles (General Oceanics) and poured into 500-mL polycarbonate bottles washed with 1.2 M HCl for analyses of chlorophyll *a*, dissolved organic carbon (DOC), and particulate organic carbon (POC). Bacterial enumeration was performed by taking 200 mL of the water sample in a polypropylene bottle and fixing immediately with glutaraldehyde at a final concentration of 1%.

Bacterial enumeration

We used 2 mL of the fixed water sample for enumerating the bacteria. Bacterial cells were stained with DAPI, filtered through black polycarbonate filters having a pore size of 0.2-µm (Millipore), and counted using an epifluorescence microscope (BX60, Olympus) under ultraviolet excitation (Porter and Feig 1980). At least 20 fields were randomly inspected in triplicate, and more than 300 bacterial cells were counted for each replicate. The length and width of each bacterial cell were measured for more than 200 bacterial cells in each sample with image analysis software (Image J; National Institute of Health). Images were captured at a magnification of 1000× with a CCD camera (ORCA-ER; Hamamatsu) equipped with an epifluorescence microscope. Bacterial cell volume was calculated as described by Nakano and Kawabata (2000).

Chemical variables

Samples for DOC were filtered through 0.2-µm polycarbonate filters (Whatman) that had been washed with 1.2 M HCl. DOC concentrations were determined using a total organic carbon analyzer (TOC-5000A; Shimadzu).

On the other hand, to determine chlorophyll *a* concentration, 100 mL to 200 mL of water samples were filtered through 0.2-µm polycarbonate filters (Whatman) and analyzed with the *N′ N*-dimethylformamide method (Moran and Porath 1980) using a fluorescence spectrometer (RF-5300PC; Shimadzu).

POC measurements were carried out between September 2010 and March 2011. Glass fiber filters of 0.3 µm nominal pore size (GF-75, Advantec) were used. About 68.7–84.9% of the bacterial cells were retained on the GF75 filter (data not shown). One to 2 L of water samples were filtered through precombusted (450°C, 5 h) GF75 filters. POC concentrations were measured using a CN corder (MT-700; Yanako). The contribution of calcium carbonate to the particulate carbon concentration was ignored because particulate calcium concentrations are

typically low in the study area (Mito et al, 2002).

RQ analysis

Water samples were filtered through the GMF2UM glass fiber filters (Whatman) to remove large particles such as phytoplankton and zooplankton, and the filtrates were filtered again through 0.2-μm Teflon filters (Advantec) to retain bacteria-sized particles. About 97.2–99.9% of bacterial cells passed through the GMF2UM filter (data not shown).

The RQ concentrations were determined using a modified method as previously described by Kunihiro et al. (2008, 2011). Briefly, quinones were extracted from the filters with a chloroform-methanol mixture (2:1, v/v) and re-extracted into hexane. UQs and MKs contained in the crude extract were separated and purified on a Sep-Pak® Plus Silica (Waters). The molecular species and concentrations of quinones were determined with a high performance liquid chromatography (HPLC) system equipped with an ODS column (pore size, 3.5 μm; Eclipse Plus C18, 3.0 × 150 mm; Agilent) and a photodiode array detector (SPD-M20A; Shimadzu). A mixture of 20% isopropylether in methanol was used as the mobile phase, at a flow rate of 0.5 mL min^{-1}. The column oven temperature was maintained at 35°C. UQs and MKs were quantified at wavelengths of 275 nm and 270 nm, respectively. UQ-10 (Sigma) was used as a quantitative standard. The quinones were identified according to their column retention times and the UV spectrum of each peak was observed in the photodiode array detector (Hiraishi and Kato 1999).

In the present paper, we refer to the RQ types using the following abbreviations: ubiquinone, UQ-*n*; menaquinone, MK-*n*. The number (*n*) indicates the number of isoprene units in the side chain of the quinone. For example, UQ-10 represents a ubiquinone with 10 isoprenoid units, and MK-9(H_2) represents a menaquinone with 9 isoprenoid units where 1 of the 9 units is hydrogenated with 2 hydrogen atoms.

161 *Cluster analysis based on the RQ profiles*

162 Quantitative evaluation of the changes in the microbial community during the study period
163 was carried out by calculating a dissimilarity index (D) based on the quinone profiling data
164 using the following equation (Hiraishi et al. 1991):

165

166 $$D(i,j) = \frac{1}{2}\sum_{k=1}^{n}\left|f_{ki} - f_{kj}\right|$$

167

168 where f_{ki} and f_{kj} are the mole fractions of the k quinone component in the i and j samples,
169 respectively. The distance matrix was used for cluster analysis.

170 The between-groups linkage method was used for cluster formation with the aid of the
171 KyPlot 5.0 program (KyensLab Inc.).

172 *Cultures*

173 Bacterial strains and a mixture of culturable bacteria were used to analyze the differences in
174 carbon yields of RQs between UQ- and MK-containing bacteria. Surface water of the
175 sampling station aged over 6 months in dark following GF/F filtration was used to prepare
176 PYG agar plates (5 g L^{-1} peptone, 2.5 g L^{-1} yeast extract, and 1g L^{-1} glucose) and 10 times
177 diluted PYG agar plates. One-hundred-microliter aliquots of water samples were spread on
178 these plates and the plates were incubated at 20ºC for 2 weeks. 4 bacterial strains, namely, O,
179 P, Y1, and Y2 (Table 2) were isolated. While colonies of the strains O and P were orange and
180 pink, respectively, the strains Y1 and Y2 were yellow with different colony morphologies
181 (Table 2). The isolates were grown axenically in diluted PYG liquid medium at 20ºC and
182 harvested in the exponential growth phase by filtration (GF75 filter; Advantec).

183 The culturable bacterial mixture was obtained by inoculating diluted PYG liquid medium
184 with 100 µl of water sample collected from the 5 m depth layer on February 2011. The
185 culturable bacteria were harvested in the same manner as the isolates. Harvested cells were

used for measuring the concentrations of quinones and POC (see above). The cell number and cell volume were estimated by fixing several milliliters of the culture medium with glutaraldehyde (final concentration, 1%). Cell-specific carbon content (CSCC), volume-specific carbon content (VSCC), and RQ-specific carbon content (RSCC) were calculated for each culture according to the following equations:

$$\mathrm{CSCC\ (fg\ C\ cells^{-1})} = \frac{\mathrm{POC\ in\ culture}}{\mathrm{Bacterial\ cell\ number\ in\ culture}}$$

$$\mathrm{VSCC\ (pg\ C\ \mu m^{-3})} = \frac{\mathrm{POC\ in\ culture}}{\mathrm{Total\ bacterial\ cell\ volume\ in\ culture}}$$

$$\mathrm{RSCC\ (mg\ C\ nmol^{-1})} = \frac{\mathrm{POC\ in\ culture}}{\mathrm{RQ\ yield\ from\ culture}}$$

Incubation experiments

Incubation experiments were conducted to determine bacterial RQ and carbon contents at the sampling site. A water sample was collected at a depth of 5 m at the sampling site on June 22, 2011, and filtered through GF/C glass fiber filters having a nominal pore size of 0.2-µm or 1.2-µm (Whatman). The 0.2-µm filtrate (microorganism free) and the 1.2-µm filtrate (bacterial grazer free) were mixed in a ratio of 9:1 to reduce bacterial density for avoiding depletion of carbon and nutrient source during the incubation and poured into a 6-L polyethylene bag. Glucose was added to each bag at a final concentration of 25 µmol L^{-1} to stimulate bacterial growth. The experiment was run in triplicate, and the experimental bags were incubated in the dark at *in-situ* temperature. After 5 days of incubation, samples were removed to measure POC concentration, bacterial quinone concentration, bacterial abundance, and cell volume. Samples for POC analysis were collected on precombusted (450°C, 5 h)

GF75 filters, and the amount of carbon was measured using a CN corder (MT-700; Yanako). CSCC, VSCC, and RSCC were also calculated from the incubation experiment.

Statistical analysis

Analyses using the Student's t-test and Pearson's coefficient were performed with Microsoft Excel.

Results

Variation in physico-chemical parameters

Water temperature at 5 m varied from June to December 2010, whereas water temperature at 70 m was low and relatively constant throughout the study period (Table 1). Because the differences in water temperature between the 5 m and 70 m depths from January to March 2011 were not more than 0.4°C, June to December 2010 was regarded as the stratification period and January to March 2011 as the mixing period. During the stratification period, DOC, POC, and chlorophyll *a* concentrations varied at 5 m, whereas at 70 m, all the parameters were relatively low with constant values (Table 1).

Seasonal variation in bacterial number, biovolume, and RQ concentration

During the stratification period, bacterial number and bacterial cell volume at 5 m varied from 1.4×10^9 to 4.3×10^9 cells L^{-1} and from 0.16 to 0.77 mm^3 L^{-1}, respectively (Fig. 1A, 1B). In contrast, the bacterial number and bacterial cell volume at 70 m were less variable (7.5×10^8 to 1.1×10^9 cells L^{-1}, 0.10 to 0.22 mm^3 L^{-1}, respectively). During the late stratification period (November or December) and the mixing period, the differences in bacterial number and cell volume between the 5 m and 70 m depths diminished (Fig. 1A, 1B).

At 5 m, the RQ concentration exhibited a variable pattern similar to that of bacterial number throughout the study period (Fig. 1C). RQ concentration at the 5 m depth gradually decreased from 101.5 pmol L^{-1} to 13.7 pmol L^{-1} during the stratification period, whereas that of the 70 m depth fluctuated between 23.0 and 42.4 pmol L^{-1}. During the mixing period, RQ

concentration of the 5 m and 70 m depths showed similar changing patterns, ranging from 14.0 to 33.0 pmol L^{-1} and from 14.7 to 25.6 pmol L^{-1}, respectively.

Significantly, the RQ concentrations at 5 m and 70 m depths showed fairly linear relationships with bacterial number ($r^2 = 0.74$, $p < 0.001$) and bacterial cell volume ($r^2 = 0.94$, $p < 0.001$) (Fig. 2). When we individually used the data of 5 m or 70m depth, we found a significant correlation only for bacterial number ($r^2 = 0.82$, $p < 0.001$) and bacterial cell volume ($r^2 = 0.98$, $p < 0.001$) at 5 m depth was observed (Fig. 2). However, there were no significant correlations at 70 m depth (Fig. 2).

Seasonal variation in UQ and MK concentrations

At 5 m, UQ and MK concentrations were the highest in July 2010 and then gradually decreased (Fig. 3A, B). At 70 m, UQ concentrations fluctuated from 9.8 to 18.9 pmol L^{-1} during the stratification period, whereas the MK concentration was relatively stable. Relative concentration of UQ was generally higher than that of MK at the hypolimnion, whereas the opposite trend was found at 5 m (Fig. 3C, D).

The major RQ concentrations at both depths showed similar changing patterns with some exceptions (Fig. 4A–F). Generally, the highest values were observed in July or August at 5 m and tended to decrease toward the mixing period. UQ-8 (Fig. 4A) and MK-9(H_8) (Fig. 4F) were the dominant RQs at 5 m, varying from 3.4 to 24.0 pmol L^{-1} and from 2.4 to 17.6 pmol L^{-1}, respectively. At 70 m, most of the dominant quinone species did not exhibit any seasonal variability, although UQ-8 highly fluctuated throughout the study period (6.0 to 17.0 pmol L^{-1}) (Fig. 4A).

During the stratification period, the relative concentrations of UQ-8 at 70 m (36% to 57%; average, 48%) was much higher than that at 5 m (24% to 33%; average, 29%) (Fig. 4G). In contrast, the relative concentrations of MK-9(H_8) at 5 m was higher (15% to 32%; average, 23%) than that at 70 m (16% to 17%; average, 17%) during the stratification period (Fig. 4L).

261 During the mixing period, the relative concentrations of UQ-8 and MK-9(H_8) were not
262 significantly different between the 5 m and 70 m depths (Fig. 4G, L).

263 The cluster analysis based on the dissimilarity of the RQ profiles divided the bacterial
264 communities into 3 different groups: Group I (5 m, stratification period), Group II (70 m,
265 stratification period), and Group III (5 m and 70 m, mixing period) (Fig. 5). Dissimilarity
266 values less than 0.1 are not recognized as different quinone profiles (Hu et al. 2001). Based on
267 these criteria, bacterial communities of Group I consist of various groups with different types
268 of RQs (>0.1).

269 The contributions of UQ-8 to the total RQ concentration of Group I, Group II, and Group
270 III were 29%, 48%, and 38%, respectively (Fig. 6). Contributions of MK-9 (H_8) to the total
271 RQ of Group I, Group II, and Group III were 23%, 7.3%, and 16%, respectively (Fig. 6).
272 UQ-9, MK-8(H_2), and MK-9(H_6) were the dominant RQs in Group I, Group II, and Group III,
273 respectively (Fig. 6).

274 *Variations in carbon content per RQ content from cultures and incubation experiment*

275 UQ-10 was detected in strains O and P, whereas strains Y1 and Y2 contained MK-6 as the
276 sole RQ (Table 2). UQ-7, UQ-8, UQ-9, and MK-7 were detected in the mixed culture at molar
277 ratios of 3:276:1:1.5 (Table 2). The average CSCC, VSCC, and RSCC were 120 fg C $cell^{-1}$,
278 0.17 pg C μm^{-3}, and 0.38 mg C $nmol^{-1}$, respectively (Table 2). The coefficients of variation
279 (CV) for CSCC, VSCC, and RSCC were 60%, 30%, and 27%, respectively (Table 2). In
280 terms of CV, the RSCC in cultures was less variable than CSCC and VSCC. The RSCC of
281 UQ-10-containing strains (average, 0.48 mg C $nmol^{-1}$) was high relative to those of
282 MK-6-containing bacteria (average, 0.33 mg C $nmol^{-1}$). However, the RSCC of the mixed
283 culture with a predominance of UQ-8 (98%) exhibited a similar value (0.30 mg C $nmol^{-1}$) as
284 that of MK-6-containing bacteria. UQ-8, UQ-10, MK-6, MK-7, and MK-10 were detected in
285 the incubation experiment at molar ratios of 27:5:52:7:1. The average CSCC, VSCC, and

RSCC from the incubation experiment were 42 fg C $cell^{-1}$, 0.17 pg C μm^{-3}, and 0.67 mg C $nmol^{-1}$, respectively.

Discussion

Bacterial biomass estimation

The coefficient of determination (r^2) for the relationship between bacterial biovolume and RQ concentration was higher (0.94) than that between bacterial number and RQ concentration (0.74), suggesting that RQ content is a better indicator of bacterial biovolume than bacterial cell number. Thus, RQs can be used for bacterial biomass estimation in Lake Biwa. However, on using the data of 70 m depth individually, RQ concentration did not correlate with cell number or biovolume (Fig. 2). The annual variation of cell number at 70 m was much lower (CV, 28%) than that at 5 m (CV, 45%), while the annual variation of cell-specific RQ concentration at 70 m was higher (CV, 40%) than that at 5 m (CV, 32%). A lack of correlation between cell abundance and RQ concentration at 70 m was probably due to the high variations of cell specific RQ concentration (Fig. 2A). The annual variation of cell volume specific RQ concentration at 70 m was also much higher (CV, 32%) than that at 5 m (CV, 14%). In the hypolimnion of Lake Biwa, the amounts of available nutrients for bacteria are much higher than that of the epilimnion during the stratification period, whereas the supply of labile DOM is limited (Nishimura et al. 2005). Bacterial nucleic acid content is affected by phosphorus concentration in the hypolimnion of Lake Biwa (Nishimura et al. 2005). The bacterial RQ content may be regulated by both the supply of organic substrates and electron acceptors such as oxygen, nitrate, and nitrite, though it is not generally understood.

The reliability of RQs as a tool for estimating bacterial carbon biomass was evaluated by examining RSCC variations in bacterial cells (Table 2). It has been reported previously that

311 cell-specific RQ content varies between strains, whereas strong positive correlations between
312 RQs and total cell number have been reported in soil and aquatic environments (Hiraishi et al.
313 2003). However, our results indicate that RQ concentration was a better indicator of bacterial
314 cell volume than cells number (Fig. 2). Thus, it is likely that the large variation in cell-specific
315 RQ content in previous studies was due to the variability of cell volumes.

316 The VSCC of freshwater bacteria in previous studies varied depending on environmental
317 conditions and bacterial activity (Nagata 1986; Bjørnsen 1986; Nagata and Watanabe 1990;
318 Kroer 1994; Loferer-Krößbacher et al. 1998). As differences in measurement techniques and
319 representativeness of the average cell volume derived from a limited number of cell size
320 measurements may result in different estimates, in contrast, the variation in RSCC in the
321 present study was small (Table 2). The RQ measurement can predict bacterial carbon biomass
322 with at least equally good precision, compared to image analysis-based measurement of
323 bacterial biovolume.

324 However, In contrast to estimations by image analysis, bacterial RQ analysis by HPLC
325 provides a less laborious way for simultaneous determination of bacterial biomass and
326 composition. About one day could be sufficient to analyze about 20 samples if the HPLC is
327 equipped with an autosampler, though the sample preparations are needed before the HPLC
328 analysis.

329 *Bacterial carbon concentration estimated by RQ concentration*

330 Individual strains grown in an artificial medium showed wide ranges of both VSCC and
331 RSCC (Table 2). As far as our knowledge goes, only a single report on RSCC value in natural
332 bacterial community: 0.43 mg C nmol RQ^{-1} (Hu et al. 2001) is available to date. However,
333 this value was derived from activated sludge samples, and the RSCC values of bacterial
334 communities in lakes have never been estimated. The RSCC value in the present study,
335 therefore, is the first estimation for planktonic bacterial biomass. The RSCC value calculated

from the incubation experiment was 0.67 mg C nmol RQ^{-1} (see Results). This RSCC value is the highest amongst the values from the strains and previous estimates, and bacterial carbon biomass estimation based on RQ concentration would carry an error ranging from 0.27 to 0.67 mg C nmol RQ^{-1} (Table. 2). However, larger variations of VSCC estimated from image analysis have been reported in a previous study and exhibited 5 fold differences within 9 analyses from natural bacterial assemblages in Lake Biwa (Nagata 1986). Such a large variation of VSCC from natural bacteria has been reported in many studies (Bjørnsen 1986, Nagata and Watanabe 1990, Kroer 1994). Thus, the estimated range of RSCC in this study is rather small, compared to the range displayed by VSCC determined by image analyses. The bacterial community composition in our incubation experiment was dominated by UQ-8, similar to natural bacterial community in the lake. Hence, the RSCC value from the natural bacterial community thus determined was used as the conversion factor for estimating planktonic bacterial carbon biomass according to the following equations: Carbon biomass (mg C l^{-1}) = 0.67 × RQ concentration (pmol L^{-1}). During the study period, the carbon biomasses of bacteria ranged from 0.008 to 0.054 mg C L^{-1} at 5 m, and from 0.010 to 0.024 mg C L^{-1} at 70 m (Table 3). Contributions of bacterial biomass to the total POC concentration at the 5 m and 70 m depths were from 3.1% to 7.1% (average: 4.7%) and from 1.9% to 10% (average: 7.4%), respectively (Table 3). Using D-amino acids as bacteria-specific biomarkers, Kawasaki et al. (2011) estimated the living bacterial contribution to POC in surface waters of North Pacific Gyre as 5.2–8.2%, which is close to the value estimated in the present study.

There are potential sources of error in the current estimation of carbon biomass. The conversion factor determined in the present study may still be an overestimation due to the presence of nonliving colloidal and submicron particles (Koike et al. 1990; Kaiser and Benner 2008), and bacterial release of nonliving detrital particles in the bacterial size fraction (Kawasaki and Benner 2006; Kawasaki et al. 2011). Further examination of the relationship

between bacterial carbon content and RQ content is needed for more reliable bacterial carbon estimation.

Succession of bacterial community structure

As there was no significant difference between RSCC of UQ-containing bacteria and that of MK-containing bacteria (Table 2), it could be assumed that RSCC would be fairly constant independent of the RQ types, and the relative concentration of individual RQ could be used as an indicator of relative biomass of individual RQ containing bacteria. Moreover, since RQs can be regarded as a specific biomarker for discriminating bacterial subgroups with different types of energy metabolism, Group I comprised highly diverse communities (>0.1) in terms of metabolic state. Group II could be further divided into several groups, although dissimilarity values were less variable than that of Group I. Bacterial communities of Group III fell into a small cluster (<0.1), except for the bacterial community at the 5 m depth in March 2011. Thus, bacterial communities of Group III are relatively uniform assemblages, suggesting that the metabolic state of bacterial communities in Group III at both the depths came similar during the mixing period.

The relative abundances of UQ-8 and MK-9 (H_8) varied among the groups (Fig. 4G, 4L) and there appear to be 2 major determinants for dividing the 3 groups in the dendrogram (Fig. 5). Bacteria with these RQs may be susceptible to changes in certain physicochemical parameters caused by water mixing and may become opportunistically dominant (Fig. 6). Chemical and biological parameters, such as DOC and chlorophyll *a* (Table 1), were almost uniformly distributed by vertical water mixing (Table 1). RQ compositions at the 2 depths also became similar during the mixing period (Fig. 5). These results suggest that bacterial groups with different types of RQs shifted due to changes in physicochemical parameters caused by vertical water mixing.

The predominant UQ-8-containing bacteria exhibited a unique oscillation pattern

particularly at 70 m throughout the study period (Fig. 4A, 4G). A broad corresponding relationship was observed between phylogenetic assignment of bacteria and dominant RQ (Collins and Jones 1981; Hiraishi 1999). *Betaproteobacteria*, which is one of the major phylogenetic bacterial groups in freshwater systems (Glöckner et al. 1999), mostly contains UQ-8 as the dominant RQ (Hiraishi 1999). UQ-8-containing bacteria have been associated with nitrogen dynamics (Sinha and Annachhatre 2007; Hamada et al. 2010), and most nitrifying, ammonia-oxidizing- and/or nitrate-oxidizing bacteria belong to this group (Lim et al. 2004; Sinha and Annachhatre 2007). A good correlation between UQ-8 concentration and nitrite + nitrate concentration ($r^2 = 0.84$, $n = 8$, $p < 0.01$) was found in the present study, although only limited data on nitrite and nitrate concentrations were available from December 2010 to March 2011 (S.D. Thottathil, unpublished data). In general, UQs are preferably produced by the nitrate respiratory types with relatively high potential electron acceptors. Particularly, UQ-8-containing bacteria may preferentially use nitrate as the final electron acceptor. However, it remains unclear whether UQ-8 is preferentially used for nitrate and nitrite respiration under oxygenated conditions, as in the hypolimnion of Lake Biwa where the annual minimum concentration of hypolimnetic dissolved oxygen is up to 3.2 mg L^{-1} (Kim et al. 2006).

Conclusion

To the best of our knowledge, this is the first study that demonstrates the utility of RQ analysis in planktonic bacterial biomass estimation. The results yielded reliable values for carbon concentration and valuable information regarding bacterial contributions to POC. Although the relative abundance of each RQ shifted due to the changes in physicochemical parameters caused by vertical water mixing, UQ-8 dominated the total RQ concentration throughout the study period and accounted for up to 57% of the total RQ concentration. Elucidation of the relationship between biomass of the major bacterial groups, such as UQ-8

411 containing bacteria, and environmental variables, along with estimation of the growth and
412 mortality of those bacterial groups in future studies may provide insights into the regulation of
413 carbon cycling by bacteria.
414
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572 Table 1. Summary of variability in physico-chemical parameters

Parameters	Stratification (5 m)	Stratification (70 m)	Mixing (5 m & 70 m)
Water temperature (°C)	22.5 ± 6.6	8.2 ± 0.1	7.7 ± 0.6
DOC (mg C L^{-1})	1.18 ± 0.07	0.93 ± 0.06	0.93 ± 0.01
POC (mg C L^{-1})	0.45 ± 0.30[a]	0.20 ± 0.04[b]	0.22 ± 0.02[c]
Chlorophyll *a* (µg L^{-1})	4.92 ± 3.81	0.30 ± 0.10	0.31 ± 0.88

573 Abbreviations: DOC, dissolved organic carbon; POC, particulate organic carbon.

574 [a] Values are from October to December 2010 (n = 3).

575 [b] Values are September, November and December 2010 (n = 3).

576 [c] Values from 70 m on March 2010 were not available due to a laboratory accident.

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Table 2. Carbon yields of bacteria and RQs from culturable bacteria

Culture	Colony features	Average cell sizes (μm^3)	Detected RQs	CSCC (fg C $cell^{-1}$)	VSCC (pg C μm^{-3})	RSCC (mg C $nmol^{-1}$)
Strain O	Orange, Small	0.62	UQ-10	150	0.24	0.43
Strain P	Pink, Small	1.3	UQ-10	220	0.17	0.52
Strain Y1	Yellow, Small	0.54	MK-6	54	0.10	0.27
Strain Y2	Yellow, Large	0.71	MK-6	120	0.17	0.39
Mix	—	0.28	UQ-7: 8: 9: MK-7 = 3: 276: 1: 1.5	46	0.17	0.30
Average				120	0.17	0.38
SD				70	0.05	0.10
CV (%)				60	30	27

Abbreviations: CSCC, cell-specific carbon content; VSCC, volume-specific carbon content; RSCC, RQ-specific carbon content.

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605 Table 3. Ranges of the estimated contributions of bacteria to POC

	5 m	70 m
Bacterial C (mg C L^{-1})[a]	0.008 – 0.054 (0.025)	0.010 – 0.024 (0.015)
Contribution to total POC (%)[b]	3.1 – 7.1 (4.7)	1.9 – 10 (7.4)

606 Abbreviations as in Table 1.

607 The values provided in parentheses are the average values.

608 [a] Values are from June 2010 to March 2011 (n = 10 [5 m] and 11 [70 m]).

609 [b] Values are from September 2010 to March 2011 (n = 7 [5 m] and 6 [70 m]).

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615 **Figure legends**

616 **Fig. 1.** Seasonal variations in (A) bacterial number, (B) bacterial biovolume, and (C)
617 respiratory quinone concentration.

618 **Fig. 2.** Relationships between (A) bacterial number and RQ concentration and (B) bacterial
619 biovolume and RQ concentration.

620 **Fig. 3.** Concentrations of (A) UQ, (B) MK, relative concentrations of (C) UQ and (D) MK at
621 water depths of 5 m and 70 m.

622 **Fig. 4.** Changes in the concentrations of (A) UQ-8, (B) UQ-10, (C) MK-7, (D) MK-8, (E)
623 MK-9, and (F) MK-9(H_8). Changes in the relative concentrations of (G) UQ-8, (H) UQ-10, (I)
624 MK-7, (J) MK-8, (K) MK-9, and (L) MK-9(H_8). Plots at July 2010 are modified from Takasu
625 et al. (2012).

626 **Fig. 5.** Cluster analysis of the dissimilarity value matrix data from mole fractions of RQs.

627 **Fig. 6.** RQ compositions of Groups I, II, and III.

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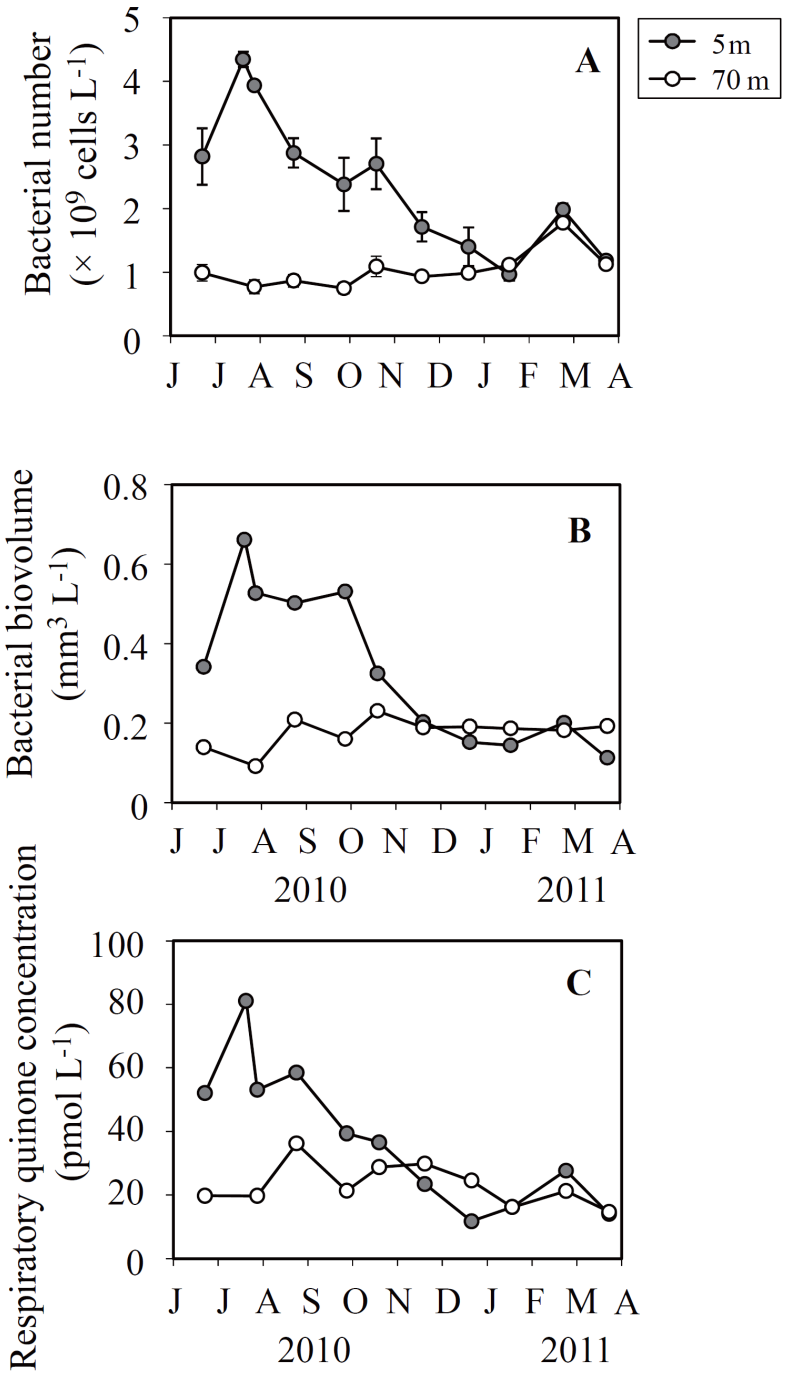

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635 Fig. 1.

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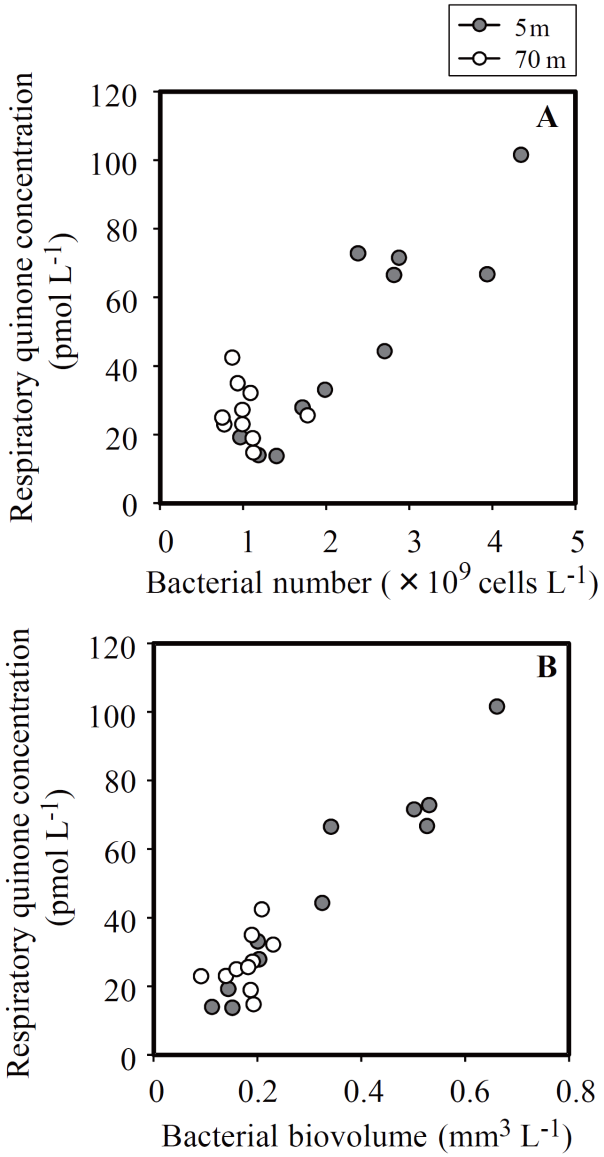


Fig. 2.

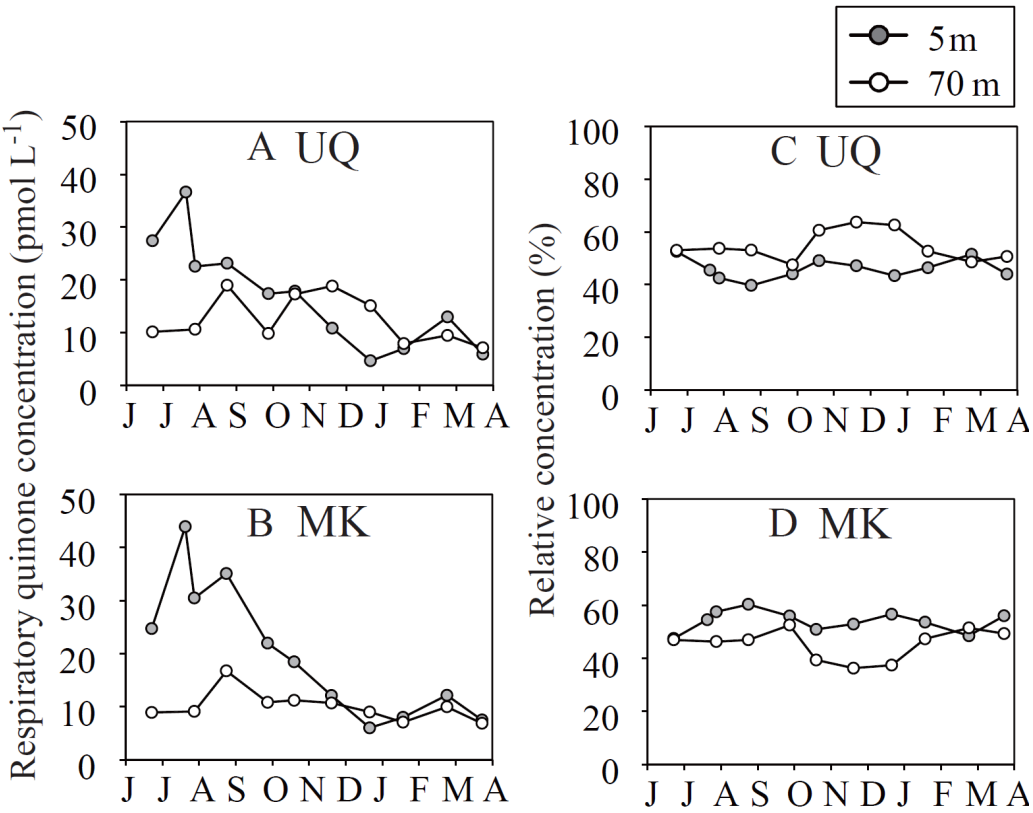
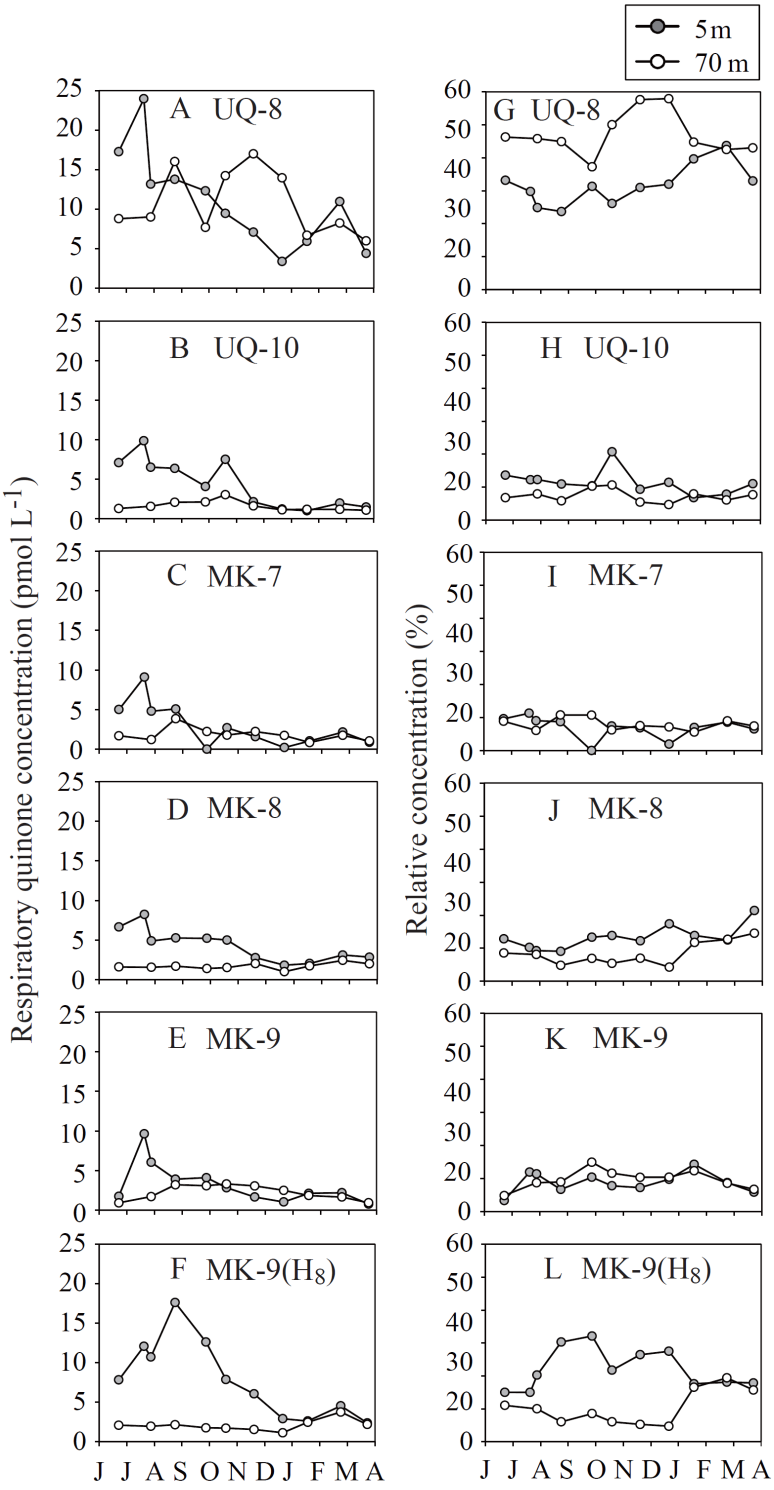


Fig. 3.



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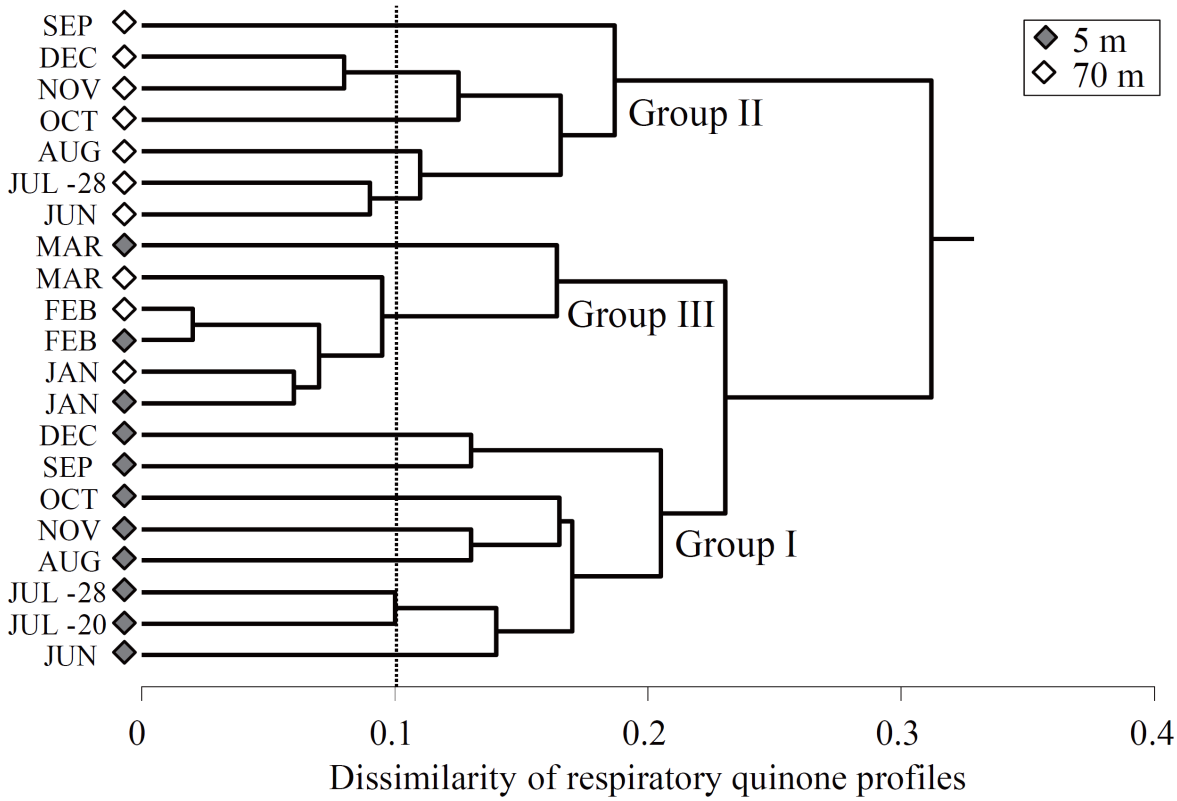

Fig. 5.

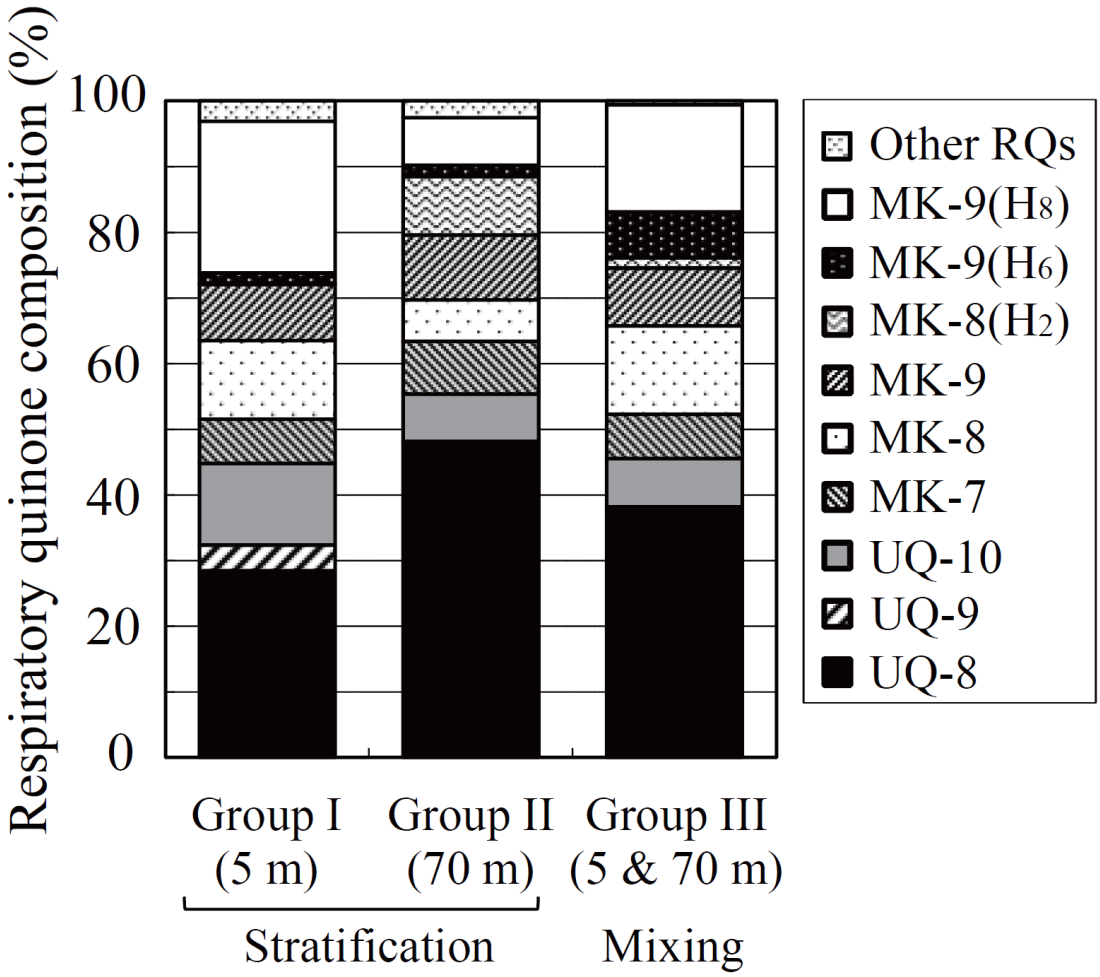

Fig. 6.